# Phosphoinositide 3-Kinase Activation in Late $G_1$ Is Required for c-Myc Stabilization and S Phase Entry[▽]

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**Phosphoinositide 3-kinase (PI3K) is one of the early-signaling molecules induced by growth factor (GF) receptor stimulation that are necessary for cell growth and cell cycle entry. PI3K activation occurs at two distinct time points during $G_1$ phase. The first peak is observed immediately following GF addition and the second in late $G_1$, before S phase entry. This second activity peak is essential for transition from $G_1$ to S phase; nonetheless, the mechanism by which this peak is induced and regulates S phase entry is poorly understood. Here, we show that activation of Ras and Tyr kinases is required for late-$G_1$ PI3K activation. Inhibition of late-$G_1$ PI3K activity results in low c-Myc and cyclin A expression, impaired Cdk2 activity, and reduced loading of MCM2 (minichromosome maintenance protein) onto chromatin. The primary consequence of inhibiting late-$G_1$ PI3K was c-Myc destabilization, as conditional activation of c-Myc in advanced $G_1$ as well as expression of a stable c-Myc mutant rescued all of these defects, restoring S phase entry. These results show that Tyr kinases and Ras cooperate to induce the second PI3K activity peak in $G_1$, which mediates initiation of DNA synthesis by inducing c-Myc stabilization.**

Exposure of quiescent cells to growth factors (GF) activates a number of early-signaling cascades involved in triggering cell cycle entry (32). Class $I_A$ phosphoinositide 3-kinase (PI3K) is a heterodimer composed of a p110 catalytic subunit and a p85 regulatory subunit, which induces phosphatidylinositol(3,4)$P_2$ [PtdIns(3,4)$P_2$] and PtdIns(3,4,5)$P_3$ formation. Class $I_A$ PI3K is one the GF-stimulated pathways that trigger S phase entry (12, 19); it is activated by Tyr kinases (Tyr-K) and Ras (15) and aids in initiating cell division by inducing cell growth and activating protein kinase B (PKB) (12). PKB inhibits glycogen synthase kinase 3 (GSK3β) and FoxO transcription factors, which in turn control cell cycle regulators (1, 22, 25, 37, 41). In addition, the expression of a constitutively active PI3K mutant augments Cdk2 activity (19). PI3K activity increases not only within minutes of GF receptor stimulation (first peak), but also in advanced $G_1$ phase (second peak) (1, 17, 38). Late-$G_1$ PI3K activity is essential for S phase entry (18, 38), but its mechanism of action remains unknown.

c-Myc also regulates cell cycle entry (3, 23, 34), and its levels are frequently increased in human cancers (30). c-Myc controls the expression of a large number of genes, including cyclin D and E and more markedly cyclin A (9, 24). c-Myc also controls Cdk kinase activity by regulating p27$^{kip}$ expression and its association with cyclin E/Cdk2 and cyclin A/Cdk2 (29, 42). c-Myc is very unstable; its stability must be precisely regulated during the cell cycle. Phosphorylation-dependent regulation of c-Myc stability involves two key residues, T58 and S62. S62 phosphorylation is mediated by microtubule-associated protein kinase (MAPK) and that of T58 by GSK3β, which targets c-Myc for degradation (43).

DNA replication requires the establishment of a replication fork. This is initiated by formation of a prereplication complex (pre-RC) that assembles when the origin replication complex is bound to the DNA replication origin, and minichromosome maintenance proteins (MCM2 to MCM7) load onto chromatin via a Cdt1- and Cdc6-dependent mechanism (4, 8, 21, 27). Binding of MCM to the origin is restricted to late mitosis and to the end of $G_1$ (in cells exiting $G_0$); following MCM loading, the origin replication complex is "licensed" for replication (21).

Activation of Cdk2 (cyclin E/Cdk2 and cyclin A/Cdk2) and Ddk (Cdc7) kinases at the $G_1$-S boundary initiates replication by recruiting Cdc45 and DNA polymerases to the origin (27). The helicase activity of the MCM complex is then required to unwind the DNA double helix (4, 8, 27). Cdc7 and Cdk2 functions are not completely defined, although many initiation components have consensus phosphorylation sites for these kinases (27). Cyclin E/Cdk2 is crucial for loading of MCM2 onto chromatin, as it cooperates with Cdc6 in pre-RC assembly; cells lacking cyclin E fail to form the pre-RC on exit from $G_0$ (11, 13). In addition, cyclin A/Cdk2 activates initiation of replication and blocks pre-RC reassembly (7).

Here, we examined the mechanism involved in PI3K activation in late $G_1$ and its role in S phase entry. To distinguish the first and second PI3K activity peaks, NIH 3T3 cells were driven into quiescence by serum deprivation and then released into $G_1$ by serum addition. This protocol allows synchronous cell cycle progression through $G_1$ and entry into S phase at approximately 9 to 12 h after serum stimulation. We show that Ras and Tyr-K activation are responsible for PI3K activation in late $G_1$. Inhibition of the late-$G_1$ PI3K activity peak did not markedly affect cyclin E levels but reduced c-Myc and cyclin A levels, Cdk2 activity, and loading of MCM2 onto chromatin. Here, we present evidence that the primary role of PI3K activity in late $G_1$ is c-Myc stabilization.

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## MATERIALS AND METHODS

**Plasmids and reagents.** The retroviral vectors encoding wild-type (WT) c-Myc–internal ribosome entry site–green fluorescent protein (GFP) or T58Ac-Myc–internal ribosome entry site-GFP (14) were kindly provided by S. Lowe (Cold Spring Harbor Laboratory, NY). pBabePuro encoding c-MycER (20) was generously donated by G. Evan (Cancer Research Institute, University of California at San Francisco, CA). Antibodies to c-Myc (C-19), cyclin E (M-20), and estrogen receptor alpha (ERα) (MC-20) were obtained from Santa Cruz Biotechnology; anti-pan-Ras and -α-tubulin were from Oncogene Research; anti-p-PKB(473) was from Cell Signaling Technologies; and anti-cyclin D3, -p27$^{Kip}$, and -MCM2 were obtained from BD Biosciences. Anti-Rb antibody was from Zymed Laboratories, anti-phospho(T58/S62)-c-Myc and anti-c-Myc (9B11) were from Cell Signaling Technologies, and anti-cyclin A and -phosphotyrosine were from Upstate Biotechnology. Monoclonal anti-β-actin antibody was from Sigma and anti-histone from Chemicon International. Horseradish peroxidase-conjugated secondary antibodies were purchased from Dako Cytomations. Enhanced chemiluminescence, L-[$^{35}$S]methionine, [α-$^{32}$P]dCTP, and [γ-$^{32}$P]ATP were from Amersham Biosciences. Lovastatin, herbimycin, and Ly294002 were from Calbiochem.

**Cell lines and cell cycle analysis.** NIH 3T3 and Phoenix cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere (5% CO$_2$, 37°C). To monitor the G$_0$-to-S transition accurately, we established a standard time course protocol for all experiments. Exponentially growing cells were seeded into dishes and rendered quiescent by incubation in DMEM-0.1% FBS (19 h). Under these conditions, cells exhibited a G$_0$ phenotype, examined as previously described (22). Quiescent cultures were rinsed with serum-free medium, and synchronous cell cycle entry was stimulated by readdition of serum (10% final concentration). Some samples were harvested immediately before serum addition (time zero). Cells were harvested at various times after serum stimulation. DNA synthesis was studied by DNA staining with propidium iodide and analyzed with a flow cytometer (Beckman-Coulter) as described previously (1) or by bromodeoxyuridine (BrdU) incorporation.

**BrdU incorporation.** Cells were incubated with 10 μM BrdU for 90 min and harvested at indicated time points by using trypsin-EDTA (see Fig. 4). Cells were washed twice with phosphate-buffered saline (PBS)-1% FBS and fixed in ice-cold 80% methanol overnight. Cells were then washed twice and resuspended in PBS containing 1% FBS and 0.1 mg/ml RNase (30 min, room temperature). To extract histones and denature cellular DNA, we incubated cells with 1.5 N HCl and 0.5% Triton X-100 (30 min, room temperature). For direct immunofluorescence staining, cells were incubated (1 h) with fluorescein isothiocyanate-conjugated anti-BrdU antibody (Becton Dickinson). After being washed (in PBS-1% FBS), cells were resuspended in 500 μl PBS (containing 0.1 mg/ml RNase, 0.1% NP-40, and 5 μg/ml propidium iodide) and analyzed by flow cytometry.

**Pulse-chase assay.** NIH 3T3 cells were incubated in DMEM-0.1% FBS for 19 h (as described above), and then the medium was replaced with Met-free RPMI medium (Gibco) containing 10% dialyzed FBS for 9 h, with 0.75 mCi [$^{35}$S]Met (each p100 dish) included for the last 6 h. At 8.5 h after serum addition, some of the samples were treated with Ly294002 (10 μM). After the 9-h incubation period described above (pulse), the [$^{35}$S]Met-containing medium was washed and replaced by DMEM-10% FBS containing 200 μM of cold Met and Cys alone or with Ly294002 (10 μM) and maintained until 12 h and 16 h after serum addition (chase).

**Inhibitor treatments and retroviral transduction.** To activate c-MycER, we added 4-hydroxytamoxifen (4-OHT) (200 nM; Sigma) 6.5 h after serum stimulation. In some cases, cells were treated with 0.1% dimethyl sulfoxide (control), lovastatin (10 μM), herbimycin (2 μM), or Ly294002 (10 μM). When samples were collected at time zero, inhibitors were added 30 min before serum addition; otherwise, inhibitors were added after 4, 6, or 7 h after serum stimulation.

Phoenix cells were transfected by Jet Pei-NaCl (Poly plus transfection) according to the manufacturer's protocols; after 10 h, cells were washed and placed in DMEM-10% FBS. Retroviral gene transduction was performed as described previously (35). Infected NIH 3T3 (c-MycER) cells were selected for 2 days in medium containing 2 μg/ml puromycin (Sigma).

**Cell lysis, immunoprecipitation, PI3K assay, WB, pull-down assay, subcellular fractionation, Northern blotting, and Cyclin/Cdk kinase assays.** Lysates were prepared in Triton X-100 lysis buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 1% Triton X-100) containing protease and phosphatase inhibitors (1 mM Na$_3$VO$_4$, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 10 μg/ml leupeptin). Immunoprecipitation, PI3K assays, and Western blotting (WB) were performed as described previously (15). For the PI3K assay, the cells

were transfected with pSG5-myc-tagged-p110α (15), and the cells were synchronized 24 h later, as described above; PI3K was immunoprecipitated using anti-Myc-tag antibody. Ras-GTP was purified from cell extracts on Sepharose-Gex2T-RBD (the Ras-binding domain of Raf-1). Briefly, NIH 3T3 cells were harvested, lysed with glutathione *S*-transferase fluorescent in situ hybridization buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 2 mM MgCl$_2$, 1% [vol/vol] NP-40, 5 mM NaF, 10% [vol/vol] glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 10 μg/ml leupeptin). Protein concentration, examined using the Micro bicinchoninic acid assay (Pierce), was normalized, and lysates were incubated (1 h, 4°C) with glutathione-Sepharose beads precoupled with glutathione *S*-transferase–RBD. Beads were washed three times in lysis buffer, and bound Ras-GTP was solubilized in 30 μl Laemmli buffer. Ras-GTP content was analyzed by WB. Total cell extracts, nuclear extracts, and chromatin fractions were isolated as described previously (26). Northern blot and cyclin/Cdk kinase assays were performed as described previously (22, 26, 35).

## RESULTS

**PI3K activity in late G$_1$ induces PKB activation that correlates with increased c-Myc protein levels.** PI3K is activated in late G$_1$; this activity peak is essential for S phase entry, since late-G$_1$ PI3K inhibition blocks S phase entry and PtdIns (3,4,5)P$_3$ addition in late G$_1$ induces cell cycle entry in the absence of serum (17, 18). To study the role of PI3K in late G$_1$, NIH 3T3 cells were driven into quiescence by serum deprivation and then released into G$_1$ by serum addition. Cells were committed to enter S phase at about 9 h after serum addition, with no further GF requirement (not shown). S phase began between 9 and 12 h after serum addition (Fig. 1A). In cells entering the cell cycle synchronously, we detected early-G$_1$ (1 h) and late-G$_1$ (~9 to 15 h) PI3K activity peaks (Fig. 1B), as determined by examining the phosphorylation of the PI3K effector PKB (p-PKB) (1). c-Myc expression levels paralleled the PI3K activity peaks (Fig. 1C).

**Ras and Tyr kinases activate PI3K in late G$_1$.** PI3K activation at G$_0$-to-G$_1$ transition is dependent on Tyr-K and Ras activities (6, 15); Ras is also activated in late G$_1$ (38). We examined Tyr-K activation by WB using an anti-pTyr antibody and Ras activity by pull-down assays. After GF addition, total Tyr-K activity increased transiently at 1 h and again between 6 and 16 h (Fig. 1D). Some of the Tyr-phosphorylated bands that appeared at 1 h differed from those visible at ~9 h, suggesting that more than one Tyr-K is activated during G$_1$ (Fig. 1D). Ras-GTP increased at ~1 h and again at 9 to 12 h after serum stimulation (Fig. 1E).

To determine whether Tyr-K or Ras stimulation is required for late-G$_1$ PI3K activation, we used small molecule inhibitors and examined the effects on p-PKB and c-Myc levels. Addition of the Ras inhibitor lovastatin (10) at 0, 4, or 6 h after serum stimulation reduced p-PKB levels at 9 and 12 h (Fig. 2A), suggesting that Ras activation is involved in late-G$_1$ PI3K activation. Addition of mevalonate, a lovastatin substrate, restored PKB phosphorylation (Fig. 2A). Herbimycin, a Src Tyr-K inhibitor (39), also reduced PKB activation at 9 and 12 h (Fig. 2A), whereas genistein, an inhibitor with high specificity for the epidermal GF receptor (40), did not affect the second G$_1$ PI3K activity peak (not shown). Combination of lovastatin and herbimycin treatments yielded a larger p-PKB reduction (Fig. 2A). The decrease in p-PKB levels correlated with a reduction in both c-Myc content and S phase entry at 12 h (Fig. 2A). The activities of the inhibitors in blocking Tyr-K and Ras activation were confirmed by WB; herbimycin reduced phos-
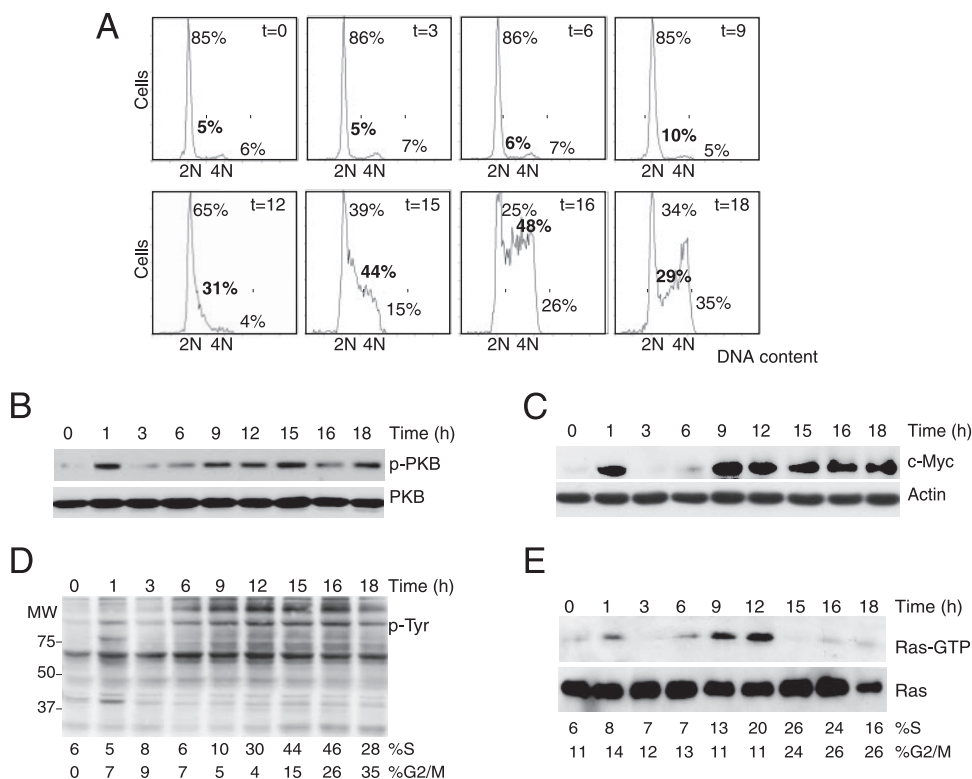
FIG. 1. Late-$G_1$ PI3K activity peak induces PKB activation that correlates with Tyr-K and Ras activation. (A) DNA content cell cycle profiles of NIH 3T3 cells arrested in $G_0$ by serum deprivation and then released for different periods (indicated) to allow synchronous cell cycle entry. Percentages of cells in $G_0/G_1$, S (in bold), or $G_2/M$ phases are indicated. (B and C) WB analysis of the cells used for panel A, using anti-phospho-S473-PKB (p-PKB), -PKB, -c-Myc, and -actin antibodies. (D) Activation of Tyr-K in cells entering cycle entry as examined by WB using anti-p-Tyr antibody. (E) Ras-GTP content was examined in cell extracts prepared as described for panel D. Total Ras and Ras-GTP were examined by WB. The figure shows data representative of at least four experiments with similar results.

pho-Tyr cellular content (Fig. 2B), and lovastatin reduced the active-Ras fraction, an effect that was reversed by mevalonate addition (Fig. 2C).

To confirm that late-$G_1$ PI3K activation requires Tyr-K and Ras, we examined PI3K activity in extracts from synchronous-cell cultures (as described above). Addition of lovastatin, herbimycin, or both at 4 h after serum addition resulted in reduced PI3K activity at 7 to 8 h after serum addition (Fig. 2D). As PI3K activity regulates cyclin levels (see below), we also examined whether lovastatin and herbimycin affected cyclin D3, E, and A levels. Tyr-K and Ras inhibition reduced the levels of these cyclins, most markedly those of cyclin A (Fig. 2E). Thus, Tyr-K and Ras cooperate in the induction of the second PI3K activity peak, which in turn regulates c-Myc and $G_1$ cyclin levels as well as S phase entry.

**Late-$G_1$ PI3K inhibition reduces c-Myc and cyclin A levels as well as Cdk2 activity.** As late-$G_1$ PI3K activation correlates with increased c-Myc expression levels, we examined the consequences of inhibiting late-$G_1$ PI3K on c-*myc* mRNA levels by Northern blotting. Cells were synchronized as described above, and the PI3K inhibitor Ly294002 was added at 7 h after serum stimulation; cells were harvested at 9 h. This analysis showed that late-$G_1$ PI3K inhibition induces a reduction of c-*myc* mRNA levels of about 15% ± 5% (at 9 h, the mean value for three experiments) (Fig. 3A), whereas c-Myc protein reduction was systematically greater than 50%. In fact, late-$G_1$ PI3K

inhibition markedly reduced c-Myc protein levels at 9 to 18 h poststimulation (Fig. 3A and B).

PI3K/PKB inhibits GSK3β (41), a kinase that phosphorylates c-Myc at Thr 58, thereby triggering Myc degradation (43). PI3K inhibition notably enhanced Thr 58-c-Myc phosphorylation, an event that correlated with c-Myc level reduction (Fig. 3B). The decrease in c-Myc protein levels correlated with the diminished expression of the Myc-transcriptional effectors cyclin D2 and Cdk4 (Fig. 3B). c-Myc stability was further examined in pulse-chase assays. Inhibition of PI3K activity in early $G_1$ (3 h after serum stimulation) blocked protein synthesis (not shown). Thus, for pulse-chase, we synchronized cells, labeled them with [$^{35}$S]Met between 3 and 9 h after serum addition, and harvested them at 9 h. At this time, the medium was replaced with nonradiolabeled Met/Cys-rich medium, alone or with Ly294002, and cells were collected at 12 and 16 h (Fig. 3C). For the sample treated with Ly294002 at the 9-h time point, the inhibitor was added 30 min before cell harvesting. PI3K inhibition greatly reduced c-Myc stability, an effect that was already evident 30 min after enzyme inhibition (Fig. 3C).

To further define the role of the second $G_1$ PI3K activity peak in S phase entry, cells were synchronized in $G_0/G_1$ and PI3K was inhibited at 7 h after serum stimulation; we examined the consequences for $G_1$-phase cyclin levels at different time points. Inhibition of late-$G_1$ PI3K activity greatly reduced cyclin A protein levels at 9 h, whereas cyclin D3 and E levels were
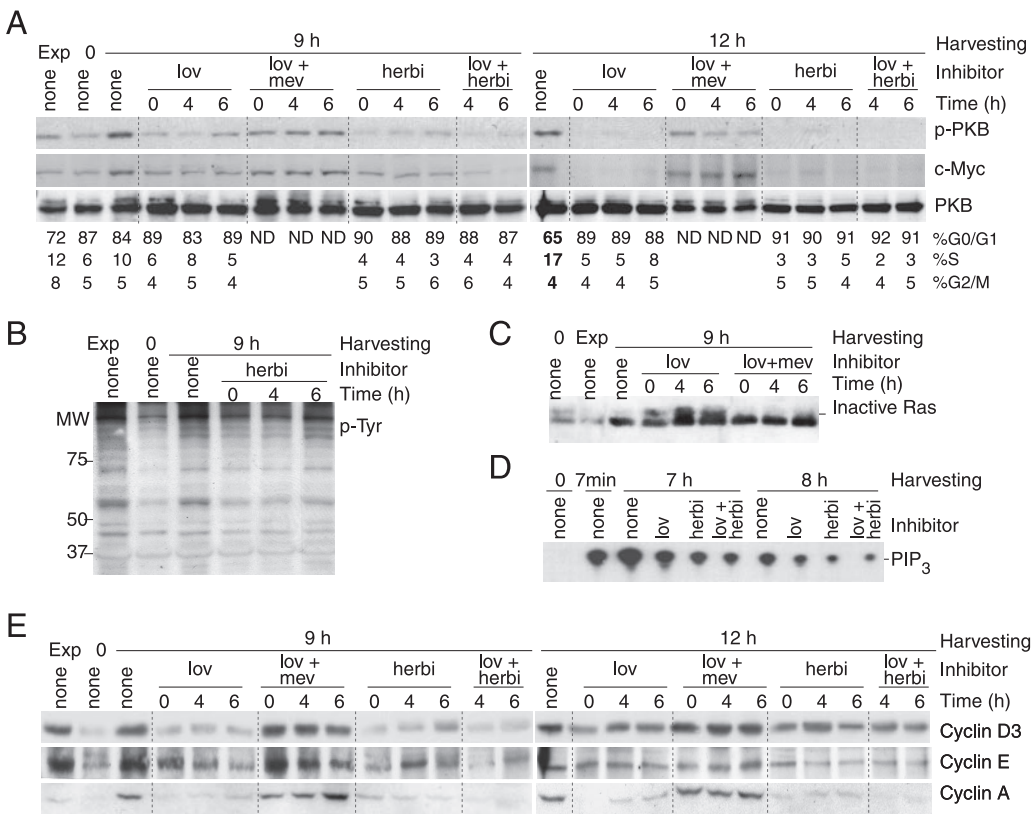
**A**

| | Exp | 0 | 9 h | | | | | | | | | | | | | 12 h | | | | | | | | | | | | | Harvesting |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | none | none | none | lov | | | lov + mev | | | herbi | | | lov + herbi | | none | lov | | | lov + mev | | | herbi | | | lov + herbi | | Inhibitor |
| | | | | 0 | 4 | 6 | 0 | 4 | 6 | 0 | 4 | 6 | 4 | 6 | | 0 | 4 | 6 | 0 | 4 | 6 | 0 | 4 | 6 | 4 | 6 | Time (h) |

p-PKB

c-Myc

PKB

| %G0/G1 | 72 | 87 | 84 | 89 | 83 | 89 | ND | ND | ND | 90 | 88 | 89 | 88 | 87 | **65** | 89 | 89 | 88 | ND | ND | ND | 91 | 90 | 91 | 92 | 91 |
| %S | 12 | 6 | 10 | 6 | 8 | 5 | | | | 4 | 4 | 3 | 4 | 4 | **17** | 5 | 5 | 8 | | | | 3 | 3 | 5 | 2 | 3 |
| %G2/M | 8 | 5 | 5 | 4 | 5 | 4 | | | | 5 | 5 | 6 | 6 | 4 | **4** | 4 | 4 | 5 | | | | 5 | 5 | 4 | 4 | 5 |

**B**

| | Exp | 0 | 9 h | | | Harvesting |
|---|---|---|---|---|---|---|
| | none | none | none | herbi | | Inhibitor |
| | | | | 0 | 4 | 6 | Time (h) |

MW — p-Tyr
75-
50-
37-

**C**

| 0 | Exp | | 9 h | | | | | | Harvesting |
|---|---|---|---|---|---|---|---|---|---|
| none | none | none | lov | | | lov+mev | | | Inhibitor |
| | | | 0 | 4 | 6 | 0 | 4 | 6 | Time (h) |

— Inactive Ras

**D**

| 0 | 7min | 7 h | | | | 8 h | | | | Harvesting |
|---|---|---|---|---|---|---|---|---|---|---|
| none | none | none | lov | herbi | lov + herbi | none | lov | herbi | lov + herbi | Inhibitor |

—PIP$_3$

**E**

| | Exp | 0 | 9 h | | | | | | | | | | | | | 12 h | | | | | | | | | | | | | Harvesting |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | none | none | none | lov | | | lov + mev | | | herbi | | | lov + herbi | | none | lov | | | lov + mev | | | herbi | | | lov + herbi | | Inhibitor |
| | | | | 0 | 4 | 6 | 0 | 4 | 6 | 0 | 4 | 6 | 4 | 6 | | 0 | 4 | 6 | 0 | 4 | 6 | 0 | 4 | 6 | 4 | 6 | Time (h) |

Cyclin D3

Cyclin E

Cyclin A

FIG. 2. Ras and Tyr kinases contribute to late-$G_1$ PI3K activation. (A) p-PKB levels in serum-starved cells, cells in exponential growth, or cells arrested in $G_0$ and then released in the presence of serum for 9 or 12 h. Lovastatin (lov), lovastatin plus mevalonate (lov+mev), or herbimycin (herbi) was added to some samples at the time of serum addition (time zero) or at 4 or 6 h after serum addition. Cell extracts were examined by WB using anti-p-PKB, -PKB, or -c-Myc antibodies. The percentages of cells in $G_0/G_1$, S, and $G_2/M$ phases are indicated. (B and C) The efficiencies of the inhibitors were tested in samples prepared as described for panel A by WB using either anti-p-Tyr (B) or anti-pan-Ras (C) antibodies. The slower-migrating, inactive Ras form is indicated. (D) PI3K was immunoprecipitated from cell extracts (see Materials and Methods) and its activity assayed in vitro. Inhibitors were added at 4 h after serum addition and cell extracts collected at the indicated time points. (E) Extracts of the cells used for panel A were examined by WB using anti-cyclin-D3, -E, and -A antibodies. (A to E) Data for one representative experiment (Exp) of four with similar results.

more affected when Ly294002 had been present for prolonged periods (Fig. 3D). The Ras inhibitor lovastatin had a greater effect than Ly294002 in reducing cyclin D3 levels (Fig. 2E and 3D); this is likely due to the Ras/MAPK dependence for cyclin D synthesis (16).

We next tested the effect of inhibiting PI3K on Cdk2 activity. The Cdk2 substrate retinoblastoma protein (Rb) was hypophosphorylated following late-$G_1$ PI3K inhibition (Fig. 3D). Consistently, both cyclin E/Cdk2 and cyclin A/Cdk2 activities decreased after PI3K inhibition (Fig. 3E). The decrease in cyclin A expression paralleled the reduction of cyclin A/Cdk2 activity. Nonetheless, late-$G_1$ PI3K inhibition affected cyclin E/Cdk2 activity more markedly than cyclin E levels. We thus examined whether PI3K inhibition reduced cyclin E/Cdk2 activity by enhancing its association with the Cdk2 inhibitor p27$^{kip}$. PI3K inhibition increased the amount of p27$^{kip}$ bound to Cdk2 (see below), explaining the reduction of cyclin E/Cdk2 activity by late-$G_1$ PI3K inhibition. Cyclin E/Cdk2 activity is required for loading of MCM2 onto chromatin (11, 13); PI3K inhibition in advanced $G_1$ resulted in a notable reduction in the amount of chromatin-bound MCM2 (Fig. 3F).

**Conditional c-Myc-ER activation rescues S phase entry in PI3K inhibitor-treated cells.** PI3K inhibition reduced cyclin A levels and Cdk2 activity. c-Myc regulates $G_1$ cyclin expression, especially that of cyclin A, and the association of p27$^{kip}$ with cyclin/Cdk2 (24, 29, 42). We thus hypothesized that the main function of late-$G_1$ PI3K activity may be to regulate c-Myc levels. To test this possibility, we used a c-Myc–estrogen receptor fusion protein (c-Myc-ER) that translocates to the nucleus after addition of an estrogen analogue such as 4-OHT (20). We examined whether the S phase entry defects induced by late-$G_1$ PI3K inhibition were counteracted by c-Myc-ER induction. Cells were infected with c-Myc-ER-expressing viruses (Fig. 4A), arrested in $G_0$, and released by serum addition. Some of the cells were treated with 4-OHT alone (at 6.5 h), with Ly294002 (at 7 h after serum addition), or with both. We collected cells at different times and examined S phase entry.

c-Myc-ER expression did not trigger S phase entry in the absence of serum (Fig. 4B and C). After serum addition, c-Myc-ER expression caused a slight increase in S phase entry compared to that in control cells, which was moderately enhanced upon 4-OHT addition (Fig. 4C). We found no notable
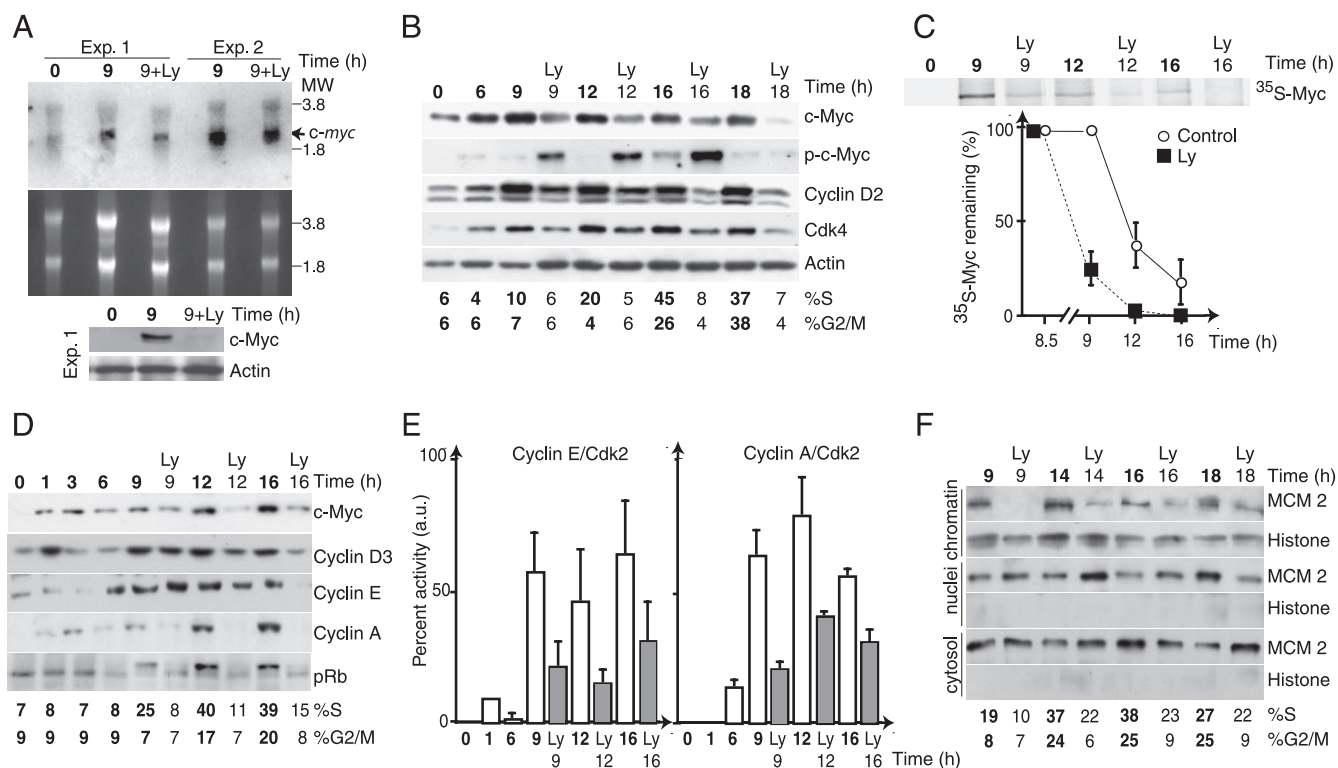
FIG. 3. Late-$G_1$ PI3K inhibition reduces c-Myc and cyclin A expression as well as Cdk2 activity. (A) Total RNA and protein extracts were prepared from cells entering the cell cycle synchronously. Ly294002 was added at 7 h after serum addition; cell harvesting was at 0 and 9 h. Samples were examined by Northern blotting (see Materials and Methods) or by WB, using a c-*myc* probe or anti-Myc antibody, respectively. The Northern blot corresponds to two different experiments (Exp.). (B) Cell extracts were prepared from cells entering the cell cycle synchronously. Ly294002 was added at 7 h after serum addition. Western blots show the expression of c-Myc, phospho-c-Myc, cyclin D2, Cdk4, and actin. The percentages of cells with sub-$G_1$, $G_0/G_1$, S, or $G_2/M$ DNA content are indicated. (C) Synchronized cells were [$^{35}$S]Met labeled during the first 9 h after serum addition, and then the medium was replaced with nonlabeled Met/Cys-rich medium alone or with Ly294002 (10 μM), and cells were collected at 12 and 16 h. For the sample treated with Ly294002 at 9 h, the inhibitor was added 30 min before harvesting. [$^{35}$S]c-Myc was examined by autoradiography. Quantitative-analysis data for three experiments are shown below the blots. (D) The cell extracts used for panel B were examined by WB using anti-c-Myc, -cyclin-D3, -E, -A, and -Rb antibodies. The percentages of cells in S and $G_2/M$ phases are indicated. (E) Cyclin E/Cdk2 and cyclin A/Cdk2 kinase activities in cyclin E and cyclin A immunoprecipitates, respectively, from cell extracts prepared as described for panel B. Histone H1 (5 μg) was used as a substrate. $^{32}$P-histone was quantitated, and the activities are represented (in arbitrary units [a.u.]). Data show means ± standard deviations for three experiments. (F) Cells entering the cell cycle synchronously, treated as described for panel B, were fractionated into cytosolic, nuclear, and chromatin fractions and were examined by WB using anti-MCM2 and anti-histone antibodies. Panels A to D and F show data for one representative experiment of three with similar results.

differences when 4-OHT was added at 0 or 6.5 h (not shown). Ly294002 treatment reduced the proportion of cells in S phase by 50%, in both c-Myc-ER- and control ER vector-expressing cells (Fig. 4C). Nonetheless, c-Myc-ER induction at 6.5 h in cells treated with Ly294002 in advanced $G_1$ (7 h) showed almost normal S phase entry levels (~85% recovery) compared to what was found for Ly294002-treated control cells (Fig. 4B and C). Induction of c-Myc-ER failed to compensate for the action of PI3K in S phase entry when PI3K was inhibited in early $G_1$ (0 to 4 h poststimulation) (Fig. 4C and data not shown). Examination of BrdU incorporation confirmed that c-Myc induction at 6.5 h counteracts S phase entry defects in cells treated with Ly294002 in advanced $G_1$ (Fig. 4D). Comparable results were obtained using the PI3K inhibitor wortmannin (not shown). These data suggest that the main function of late-$G_1$ PI3K activity is to regulate c-Myc protein levels.

**Expression of a GSK3β-resistant c-Myc mutant rescues the cell cycle entry defects induced by inhibiting late-$G_1$ PI3K activity.** PI3K/PKB inactivate GSK3β, an enzyme that targets

c-Myc for degradation (43). To confirm that c-Myc stabilization is the main role of PI3K activity in late $G_1$, we examined the effect of inhibiting PI3K in cells expressing the c-Myc$_{T58A}$ substitution mutant, which is resistant to GSK3β action (14).

Cells were transfected with GFP control vector or with cDNAs encoding GFP fused to wild-type (WT) c-Myc or c-Myc$_{T58A}$ (Fig. 5A). Transfected cells were sorted, and cultures were synchronized, released from arrest, and treated with Ly294002 at 7 h after serum addition. Cells were harvested at different time points. Overexpression of either WT c-Myc or c-Myc$_{T58A}$ induced apoptosis and cell cycle entry in the absence of serum (Fig. 5B). Late-$G_1$ PI3K inhibition reduced cell cycle entry in control cells and to a lesser extent in cells overexpressing WT c-Myc; c-Myc$_{T58A}$ expression, however, largely restored cell cycle entry (Fig. 5B). To reduce c-Myc expression levels, cells were infected with viruses encoding c-Myc$_{T58A}$. Under these conditions, c-Myc$_{T58A}$ did not significantly induce cell cycle entry in the absence of serum (Fig. 5C). Synchronous-cell-infected cultures were treated with Ly294002 at 7 h
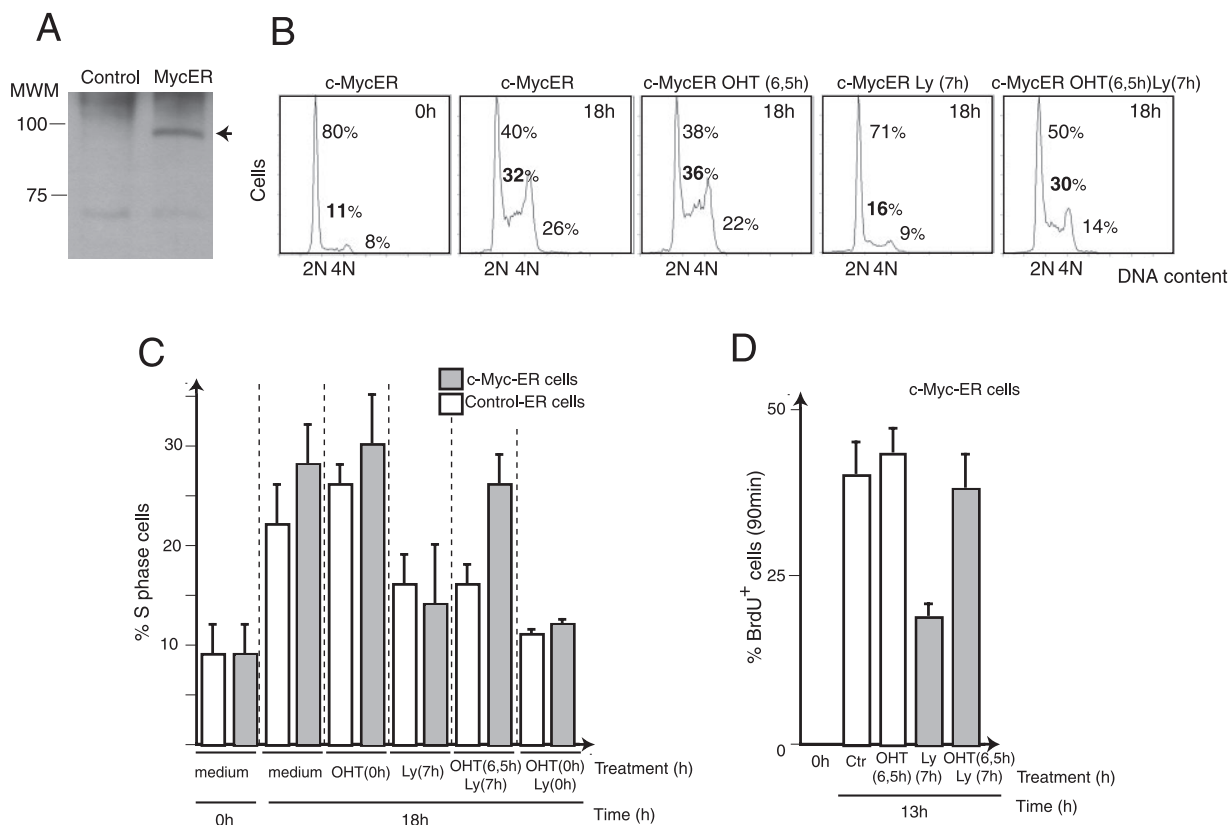
FIG. 4. Conditional activation of c-Myc-ER rescues S phase entry blockade induced by late-G$_1$ PI3K inhibition. (A) c-Myc-ER expression was examined in NIH 3T3 c-Myc-ER-infected cells by WB using anti-Myc antibody. (B) Cell cycle profiles of c-Myc-ER cells in quiescence (time zero) or at 18 h after serum addition, alone or in the presence of OHT (200 nM; added at 6.5 h after serum addition) and/or Ly294002 (10 μM; added at 7 h after serum addition). The figure shows results for one representative experiment of three performed. (C) Percentages of c-Myc-ER and control cells in S phase. Cells were treated as described for panel B. Data for a sample of cells treated with OHT (200 nM) and Ly294002 (10 μM) at the time of serum addition (time zero) are included. Data shown are mean values for three experiments. (D) BrdU incorporation in NIH 3T3 c-Myc-ER-infected cells entering the cell cycle synchronously as in panel B and collected at 13 h after serum addition. BrdU (10 μM) was present for the last 90 min. Data shown are means ± standard deviations for three experiments.

after serum addition and harvested at different time points. PI3K inhibition blocked cell cycle entry in control cells, but cell cycle entry was nearly normal in cells expressing c-Myc$_{T58A}$ (Fig. 5D). These results indicate that a stable form of c-Myc substitutes for PI3K action in late G$_1$.

**c-Myc$_{T58A}$ expression rescues cyclin A expression, Cdk2 activity, and MCM2 loading defects induced by late-G$_1$ PI3K inhibition.** To confirm that the primary effect of PI3K activity in advanced G$_1$ is to stabilize c-Myc, we examined whether c-Myc$_{T58A}$ expression compensated for the cell cycle entry defects induced by late-G$_1$ PI3K inhibition. PI3K inhibition moderately affected cyclin D3 and E expression levels (see above). Similarly, c-Myc$_{T58A}$ expression did not markedly alter cyclin D3 (not shown) or cyclin E levels (Fig. 6A). In contrast, cyclin A expression levels were greatly reduced upon late-G$_1$ PI3K inhibition (Fig. 6A). c-Myc$_{T58A}$ expression increased cyclin A expression in Ly294002-treated cells and moderately increased basal cyclin A levels (Fig. 6A). Moreover, whereas hyperphosphorylated Rb levels, cyclin E/Cdk2, and cyclin A/Cdk2 kinase activities were reduced by late-G$_1$ PI3K inhibition in control cells, they were virtually unaffected in c-Myc$_{T58A}$-expressing cells (Fig. 6A to C). As c-Myc controls the levels of Cdk2 bound to p27$^{kip}$ (29, 42), we tested whether

p27$^{kip}$-Cdk2 association was affected by c-Myc$_{T58A}$ expression. Ly294002 treatment at 7 h in synchronous-cell cultures increased the association of p27$^{kip}$ with cyclin E/Cdk2 in controls, but association was lower and resistant to PI3K inhibition in c-Myc$_{T58A}$-expressing cells (Fig. 6D). Similar results were obtained using c-Myc-ER-expressing cells treated with 4-OHT (at 6.5 h), Ly294002 (at 7 h), or both simultaneously (Fig. 6E and data not shown). In fact, c-Myc-ER induction corrected the defects in S phase entry, cyclin A expression, and Rb phosphorylation induced by late-G$_1$ PI3K inhibition (Fig. 6E).

We also examined the consequences of expressing c-Myc$_{T58A}$ on the loading of MCM2 onto chromatin. In control cells, MCM2 loading was still low at 9 h (similar to that observed at 0 h), increased at 12 to 16 h, and was blocked by PI3K inhibition. In contrast, in c-Myc$_{T58A}$ cells, MCM2 loading increased by 9 h and remained insensitive to late-G$_1$ PI3K inhibition (Fig. 7).

## DISCUSSION

Activation of PI3K in late G$_1$ is essential for cell cycle entry (1, 12, 17). We examined the signals involved in late-G$_1$ PI3K activation and the mechanisms by which this event controls the G$_1$-to-S transition. We report that tyrosine kinase and Ras
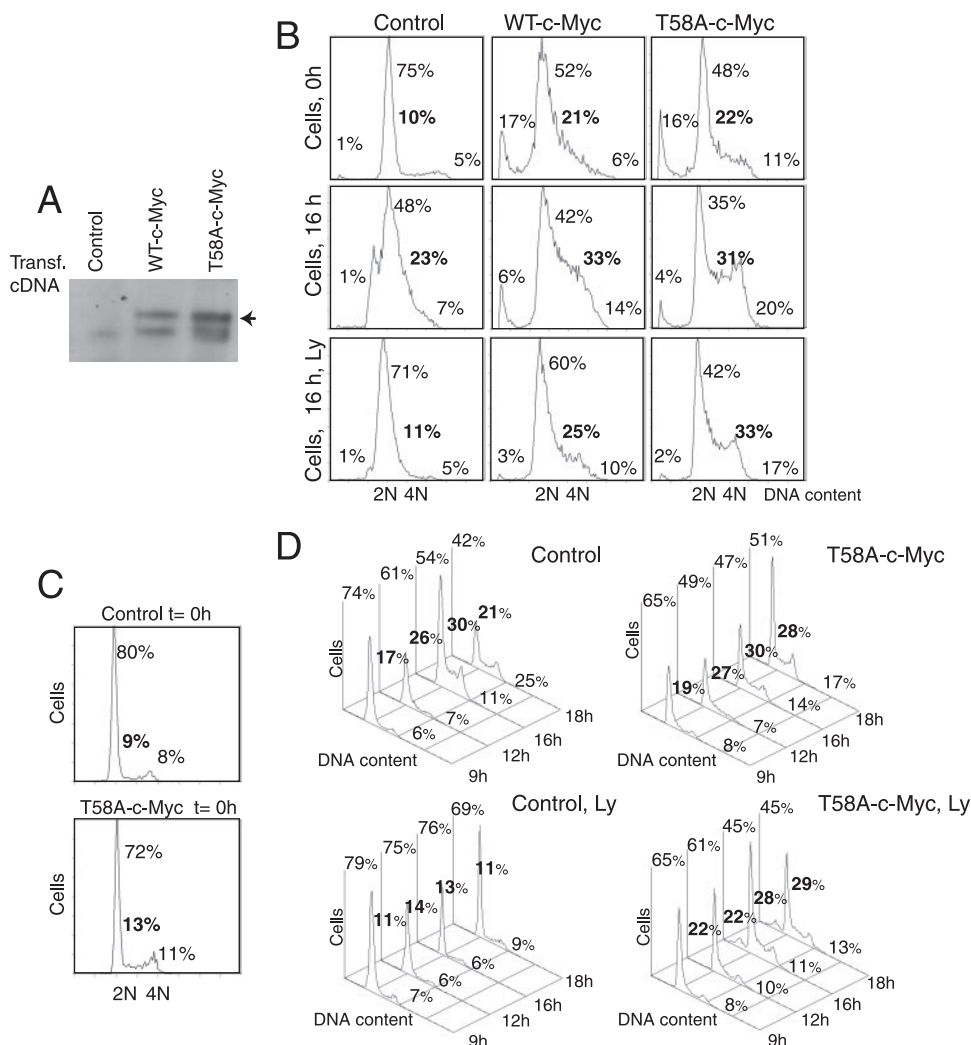
FIG. 5. Expression of c-Myc T58A rescues cell cycle entry defects induced by late-$G_1$ PI3K inhibition. (A) WT c-Myc and c-Myc$_{T58A}$ expression in NIH 3T3 cells, tested by WB using anti-Myc antibody. (B) DNA content in representative NIH 3T3 cells transfected (Transf.) with a control vector or cDNA encoding WT c-Myc or c-Myc$_{T58A}$. Cells were arrested by serum deprivation (time zero) and released by serum addition for 16 h. Some samples were incubated with Ly294002 added 7 h after serum stimulation. (C and D) DNA content in representative NIH 3T3 cells infected with a control virus or a virus encoding c-Myc$_{T58A}$. Infected cells were arrested by serum deprivation (C) or first arrested and then released by serum addition for 9, 12, 16, and 18 h (D). Some samples were incubated with Ly294002 added 7 h after serum stimulation. (A to D) Data representative of one experiment of four with similar results. Percentages of cells in $G_0/G_1$, S (in bold), and $G_2/M$ phases are indicated.

activation are required to induce the PI3K/PKB pathway in late $G_1$. Since PI3K/PKB inactivates GSK3β, the enzyme that targets c-Myc for degradation (41, 43), we hypothesized that late-$G_1$ PI3K activation may be essential for c-Myc stabilization. We observed that PI3K inhibition in advanced $G_1$ decreases c-Myc and cyclin A levels, reduces cyclin E/Cdk2 and cyclin A/Cdk2 activity, and increases the fraction of p27$^{kip}$ bound to cyclin E/Cdk2; c-Myc-deficient cells show these defects (23, 24, 42). Moreover, c-Myc induction in late $G_1$ and the expression of a c-Myc mutant (c-Myc$_{T58A}$) (14) that does not require PI3K/PKB for stabilization counteract the cell cycle entry defects induced by PI3K inhibition in late $G_1$, including those related to DNA synthesis, cyclin A expression, cyclin E/Cdk2 and cyclin A/Cdk2 activity, and the association of p27$^{kip}$ with cyclin E/Cdk2. We conclude that c-Myc stabilization is a major role for PI3K activation in late $G_1$.

c-*myc* mRNA and protein both have very short half-lives (20 to 30 min). To achieve the c-Myc expression levels required for cell cycle entry, c-Myc stability must be regulated during $G_1$ (9, 30); we show that late-$G_1$ PI3K activation stabilizes c-Myc. This conclusion is further supported by our in-progress studies using interfering RNA and constitutive active mutants of class $I_A$ PI3K isoforms. Although these tools do not allow distinction between the first and second PI3K activity peaks in $G_1$, they confirm the role of PI3K in cell cycle entry and in c-Myc expression control. Whereas activation of PI3K accelerates cell cycle entry and increases c-Myc levels, decrease of PI3K levels reduces S phase entry and c-Myc content (not shown).

Phosphorylation-dependent regulation of c-Myc stability involves two key residues, T58 and S62. MAPK mediates S62 phosphorylation, which stabilizes c-Myc, but is required for subsequent T58 phosphorylation by GSK3β, which then in-
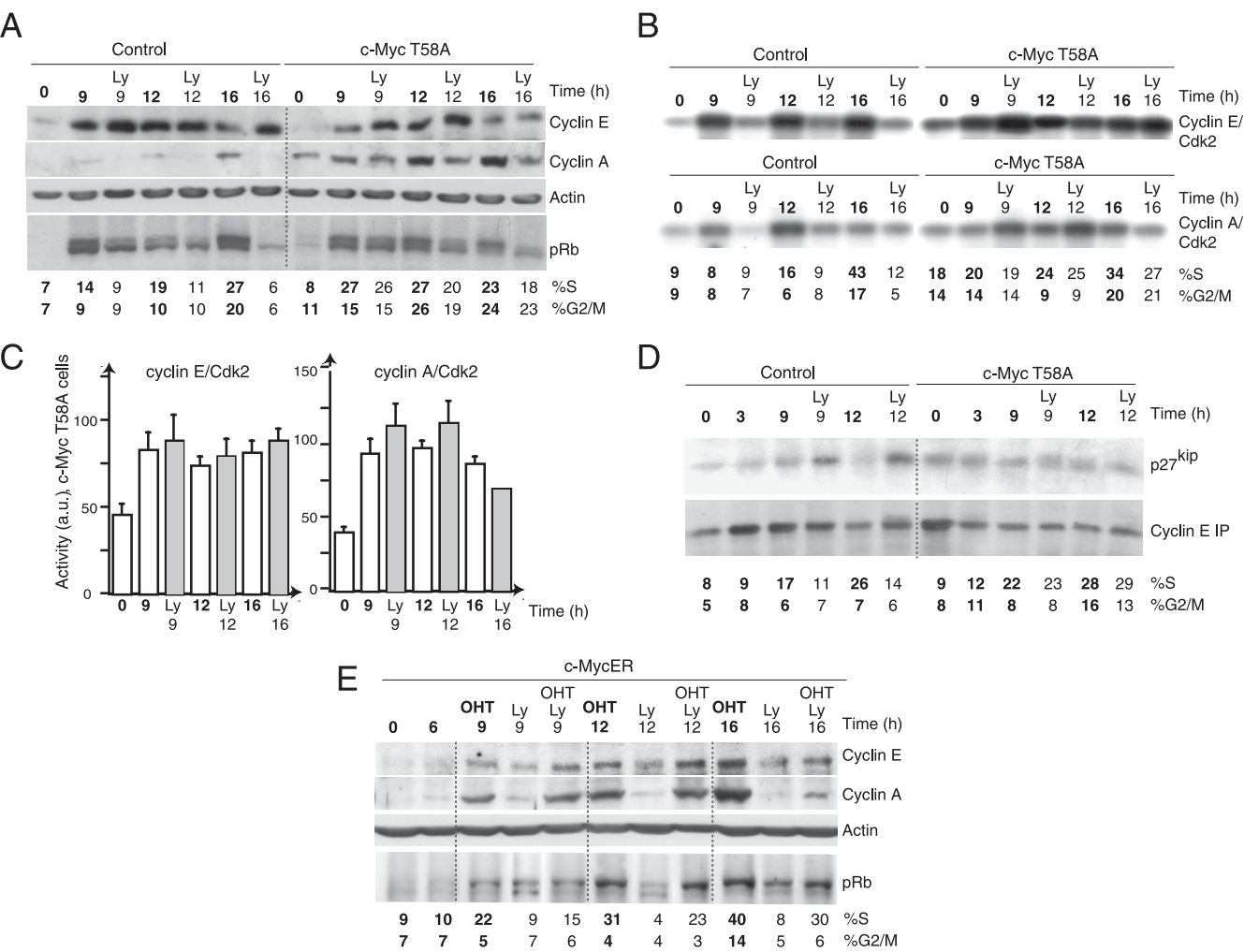
FIG. 6. c-Myc$_{T58A}$ expression counteracts the cyclin A expression, Cdk2 activity, and MCM2 chromatin loading defects induced by late-G$_1$ PI3K inhibition. (A) Control and c-Myc$_{T58A}$-expressing cells were arrested in G$_0$ and then released and treated with Ly294002 at 7 h after serum addition. Cells were harvested at different time points (indicated) and extracts examined by WB using anti-cyclin E, -cyclin A, -actin, and -Rb antibodies. (B) Cyclin E/Cdk2 and cyclin A/Cdk2 kinase activities in cyclin E and cyclin A immunoprecipitates, respectively, of cell extracts from control and c-Myc$_{T58}$-expressing cells treated as described for panel A. Cdk2 activity was measured as described in the legend to Fig. 3. (C) Cyclin E/Cdk2 and cyclin A/Cdk2 activities in c-Myc$_{T58A}$-expressing cells were examined by in vitro kinase assays performed as described for panel B. Data shown are means ± standard deviations for three experiments. (D) The cells used for panel A were lysed, and cyclin E was immunoprecipitated from lysates (200 μg). Samples were resolved and examined by WB using anti-p27$^{Kip}$ or anti-cyclin E antibody. (E) Synchronized c-Myc-ER-expressing cells were treated with 4-OHT (at 6.5 h), Ly294002 (at 7 h), or both simultaneously; cells were collected at different time points (indicated). Cyclin E, cyclin A, pRb, and actin levels were examined by WB. (A, B, D, and E) Data for one representative experiment of three with similar results. Percentages of cells in S and G$_2$/M phases are indicated.

duces c-Myc degradation (43). T58 phosphorylation nonetheless appears to be the key destabilizing event, as it represents a major mutation hot spot in Burkitt's lymphomas (14). Since S62 phosphorylation is a prerequisite for T58 phosphorylation, c-Myc might also be phosphorylated in late G$_1$ by MAPK, as we observed concomitant activations of MAPK and PI3K at this point (not shown).

c-Myc function is linked to its transcription factor activity, which is required for its transforming capacity (3, 9, 30). c-Myc regulates transcription by association with the Max protein. Gene expression regulated by c-Myc/Max involves several mechanisms that include chromatin remodeling as well as recruitment of RNA polymerases and transcription elongation factors (9, 28). c-Myc regulates the expression of a number of

target genes, including cyclins D and E and, to a large extent, cyclin A (30). The first c-Myc expression peak in G$_1$ occurs at ~1 h after serum stimulation. Since c-Myc promotes cyclin D and E expression (24), the first c-Myc expression peak may trigger the expression of these cyclins in early/mid-G$_1$. Later on, c-Myc is essential for cyclin A expression, as well as for inhibiting the association of p27$^{kip}$ with Cdk2 complexes (24, 29, 42). The kinetics of cyclin A expression, its reduction following c-Myc destabilization (by late-G$_1$ PI3K inhibition), and the association of p27$^{kip}$ with cyclin E/Cdk2 complexes suggest that the second c-Myc expression peak regulates cyclin A induction and Cdk2 activity by controlling its association with p27$^{kip}$ (29, 42).

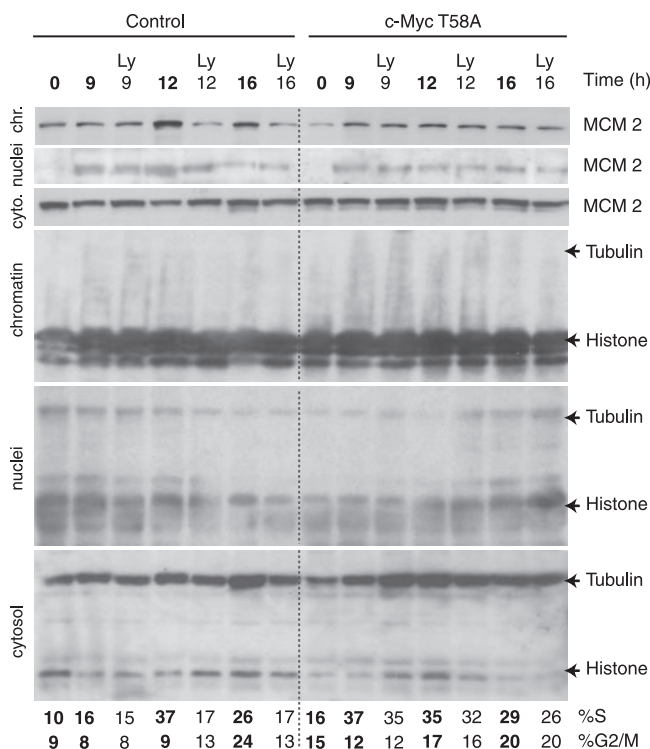Although late-G$_1$ PI3K action is nearly identical to that of

FIG. 7. c-Myc$_{T58A}$ expression counteracts the reduced MCM2 chromatin loading induced by PI3K inhibition in late G$_1$. Western blots documenting MCM2 protein levels in different subcellular fractions (as shown in Fig. 3F) of cells treated as described in the legend to Fig. 6A are shown. WB analyses using anti-tubulin and -histone antibodies were used as controls for fraction purity.

c-Myc, PI3K and c-Myc have otherwise unrelated functions and appear to cooperate for cell cycle entry (17), suggesting distinct functions. PI3K/PKB activation is required for inactivation of FoxO transcription factors, which inhibit the expression of several c-Myc targets, providing a mechanism for the cooperative action of c-Myc and PI3K in early G$_1$ (5). In fact, inhibition of PI3K in early G$_1$ (during the first 6 h) impaired cell growth and cycle entry, and this blockade was not counteracted by c-Myc-ER induction (Fig. 4C). In early G$_1$, PI3K regulates cell growth, FoxO transcription factor inactivation, and GSK3β inhibition, events that in turn control cyclin D levels (1, 2, 22, 25, 31, 33, 36). Nonetheless, Tyr-K and Ras are reactivated in mid-/late G$_1$, driving PI3K activation (Fig. 2). We show that the main role of late-G$_1$ PI3K activity is to stabilize c-Myc. Stabilized c-Myc in turn triggers cyclin A synthesis, reduces the binding of p27$^{kip}$ to Cdk2 complexes, and induces cyclin E/Cdk2 (which regulates MCM2 loading) and cyclin A/Cdk2 activities. These events are crucial for DNA synthesis induction, explaining the requirement for PI3K activity in late G$_1$ for cell cycle entry.

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